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Heterogeneous Mutations in the Glucose-6-Phosphatase Gene in Japanese Patients With Glycogen Storage Disease Type Ia

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Glycogen storage disease type Ia (GSD-Ia) is an autosomal recessive disorder of glycogen metabolism caused by glucose-6-phosphatase (G6Pase) deficiency. It is characterized by short stature, hepatomegaly, hypoglycemia, hyperuricemia, and lactic acidemia. Various mutations have been reported in the G6Pase gene (*G6PC*). However, in Japanese patients, a g727t substitution was found to be the major cause of GSD-Ia, accounting for 20 of 22 mutant alleles [Kajihara et al., 1995], and no other mutations have been found in this population. We analyzed four Japanese GSD-Ia patients and identified three other mutations in addition to the g727t. They included two missense mutations (R83H and P257L) and one nonsense mutation (R170X). Each of the three mutations exhibited markedly decreased G6Pase activity when expressed in COS7 cells. A patient homozygous for R170X showed multiple episodes of profound hypoglycemia associated with convulsions, while P257L was associated with a mild clinical phenotype. The presence of R170X in three unrelated families may implicate that it is another important mutation in the etiology of GSD-Ia in Japanese patients. Thus, the detection of non-g727t mutations is also important in establishing the DNA-based diagnosis of GSD-Ia in this population. Am. J. Med. Genet. 92:90–94, 2000.

KEY WORDS: glycogen storage disease type Ia; glucose-6-phosphatase gene; allelic heterogeneity; DNA diagnosis

INTRODUCTION

Glycogen storage disease type Ia (GSD-Ia) is an autosomal recessive disorder of glycogen metabolism [Chen and Burchell, 1995]. It has been identified panethnically with an incidence of approximately one in 100,000 to 300,000 live births [Lei et al., 1993]. GSD-Ia is caused by a deficiency of microsomal glucose-6-phosphatase (G6Pase, EC 3.1.3.9) in the liver, kidney, and intestinal mucosa, leading to excessive accumulation of glycogen in these organs. Clinical manifestations include short stature, hepatomegaly, hypoglycemia, hyperuricemia, hyperlipidemia, and lactic acidemia. Patients with GSD-Ia often suffer from hepatic adenoma/hepatoma, renal dysfunction, and pulmonary hypertension, all of which can be fatal. Although these clinical characteristics are highly informative for the diagnosis of GSD-Ia, the definitive diagnosis has been based on the result of enzymatic assay of G6Pase in a biopsied liver specimen in order to clearly distinguish GSD-Ia from type GSD-Ib [Narisawa et al., 1978]. The GSD-Ib is associated with slightly different clinical profiles as well as different therapeutic requirements.

The human G6Pase gene (*G6PC*) spans approximately 12.5 kb on chromosome 17 and contains five exons [Lei et al., 1993]. The hydropathy profiles deduced from amino acid sequences predict that G6Pase contains nine transmembrane helices, with its amino and carboxyl termini facing the endoplasmic reticulum lumen and the cytoplasm, respectively [Pan et al., 1998]. Molecular analysis of GSD-Ia patients has revealed 29 mutations [Human Gene Mutation Database: http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html], although a polymorphic T192A is also included [Okubo et al., 1997]. These studies identified prevalent mutations in various ethnic groups; R83C, G347X, two-base insertion in exon 3, and R83H account for 41, 35, 50, and 70% of mutant alleles in Caucasian, French, Hispanic, and Chinese patients, respectively [Lei et al.,

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1995a; Chevalier-Porst et al., 1996]. In Japanese patients, a g727t substitution was found to be the major cause of GSD-Ia, accounting for 23 (92%) of 25 mutant alleles [Kajihara et al., 1995; Okubo et al., 1997], suggesting that this mutation is predominant in Japanese patients and that screening for the substitution would be diagnostic. Here we report three other mutations in *G6PC* among four Japanese patients with GSD-Ia.

MATERIALS AND METHODS

Patients

Four Japanese patients with GSD-Ia referred to Tohoku University Hospital for diagnosis and treatment underwent molecular analysis. Parents of patients 1, 2, and 4 were not related, whereas those of patient 3 were first cousins. Patients 1, 2, and 4 showed marked hepatomegaly in infancy, which was associated with short stature, hypoglycemia, hyperuricemia, hyperlipidemia, and lactic acidemia. Recurrent convulsions caused by severe hypoglycemia was observed in patient 1. Diagnosis of GSD-Ia was made in patient 3 at age 26 years when hyperuricemia was noted upon examination for her joint pain. She also had hyperlipidemia, lactic acidemia, and mild hypoglycemia after prolonged fasting. Her height was in the normal range. Diagnosis of each patient was made by the measurement of G6Pase activity in biopsied liver specimens [Narisawa et al., 1983]. The residual G6Pase activities were 0.09, 0.32, 0.6, and 0.02 mmol Pi/min/g liver in patients 1, 2, 3, and 4, respectively (normal values: 6.26 ± 1.22 mmol Pi/min/g liver).

Molecular Analysis

Genetic analysis was performed with informed consent of the patients and/or their parents. Genomic DNA was extracted from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines established from the patients. Five exons of *G6PC* were amplified by polymerase chain reaction (PCR) as described by Lei et al. [1993]. Amplified PCR fragments were separated on a 1.0% SeaPlaque low melting agarose gel (FMC BioProducts, Rockland, ME), purified by QIAEX Gel Extraction Kit (Qiagen, Hilden, Germany), and subcloned into ddT-tailed pBluescript II KS(+) (Stratagene, La Jolla, CA) [Holton and Graham, 1991]. At least five clones of each fragment were isolated and the plasmids were sequenced on an automated sequencer (ALFexpress, Pharmacia Biotech, Sweden) using an AutoRead Sequencing Kit (Pharmacia Biotech). Direct sequencing of PCR fragments was also performed [Kure et al., 1998]. A nucleotide polymorphism at nt 1,176 in the 3′-UTR of *G6PC*-cDNA was examined by *Dra*I digestion of PCR products as described previously [Wong et al, 1998].

The wild-type *G6PC*-cDNA was subcloned into a mammalian expression vector pUC-CAGGS [Niwa et al., 1991], which carries the chicken β-actin gene promoter and cytomegalovirus enhancer. In vitro site-directed mutagenesis was performed on the cDNA subcloned in pUC118 to produce the mutant cDNA with a previously reported method [Deng and Nickoloff, 1992]. Each mutated cDNA was then subcloned into an *Eco*RI site of pUC-CAGGS. COS7 cells were transfected by electroporation with each of the recombinant vectors and pSV-β-galactosidase (Promega, Madison, WI), which expresses *Escherichia coli* β-galactosidase under the control of the SV40 early promoter and enhancer. After 48 hr, the cells were harvested and the G6Pase activity was measured essentially as described previously [Narisawa et al., 1983].

RESULTS

The sequencing of genomic DNA from the four patients revealed four nucleotide substitutions: "g-to-a" transition at nucleotide position (nt) 327 (g327a), c588t, c849t, and g727t. The first three mutations lead to amino acid substitutions, R83H, R170X, and P257L, respectively (Fig. 1), whereas g727t was the same mutation as reported previously to cause aberrant mRNA splicing [Kajihara et al., 1995]. The R170X and R83H produce a new *Bsp*HI and a *Fok*I recognition site in mutant allele, respectively. The P257L can be detected by *Eco*NI digestion of a PCR fragment amplified with a primer set (5′–3′) of ATATTTTCTCATTACCTTCTTCCTG/TTCTTGAGGAGCCTGGCAAA. The presence of g727t is examined by *Rsa*I digestion of a PCR product with a primer set of TGCTTTCTTCCACTCAGGCA/AATCCGATGGCGAAGCTGTA. The mutant allele is not digested but the wild-type allele is cut by the enzyme. Sequencing of multiple plasmid clones as well as direct sequencing indicated that patient 1 is a homozygote for R170X, patient 2 a compound heterozygote for R83H and g727t, patient 3 a homozygote for P257L, and patient 4 a compound heterozygote for R170X and g727t. Patient 2 has a 1176T polymorphic allele on the R83H-carrying chromosome and a 1176C allele on the g727t-carrying chromosome. The presence of each mutation and genotypes of four patients were confirmed by the restriction analysis (Fig. 2). Both parents of patient 1 are heterozygous for R170X. Patient 2 inherited the R83H allele from her mother and the g727t allele from her father. Both parents of patient 3 were heterozygotes for P257L.

The G6Pase activity in COS7 cells, which were transfected with R83H-, P257L-, or R170X-carrying recombinant vectors was dramatically reduced, compared to the activity using wild-type cDNA (Table I). This indicated that these mutations are directly involved in the etiology of GSD-Ia. Among the three mutations, P257L showed relatively higher residual activity (1.2%, 1.87 ± 0.7 nmol Pi/mg protein/min) than R170X (0.3%, 0.45 ± 0.33 nmol Pi/mg protein/min), when the activity of the wild-type allele is 100% (157.9 ± 55.8 nmol Pi/mg protein/min) ($P < 0.05$).

DISCUSSION

We identified four mutations (R170X, P257L, R83H, and g727t) of *G6PC* in all four Japanese GSD-Ia patients examined. Patient 1, a boy homozygous for R170X, had recurrent episodes of severe hypoglycemia from infancy. These clinical symptoms were not observed in three other patients, suggesting that R170X

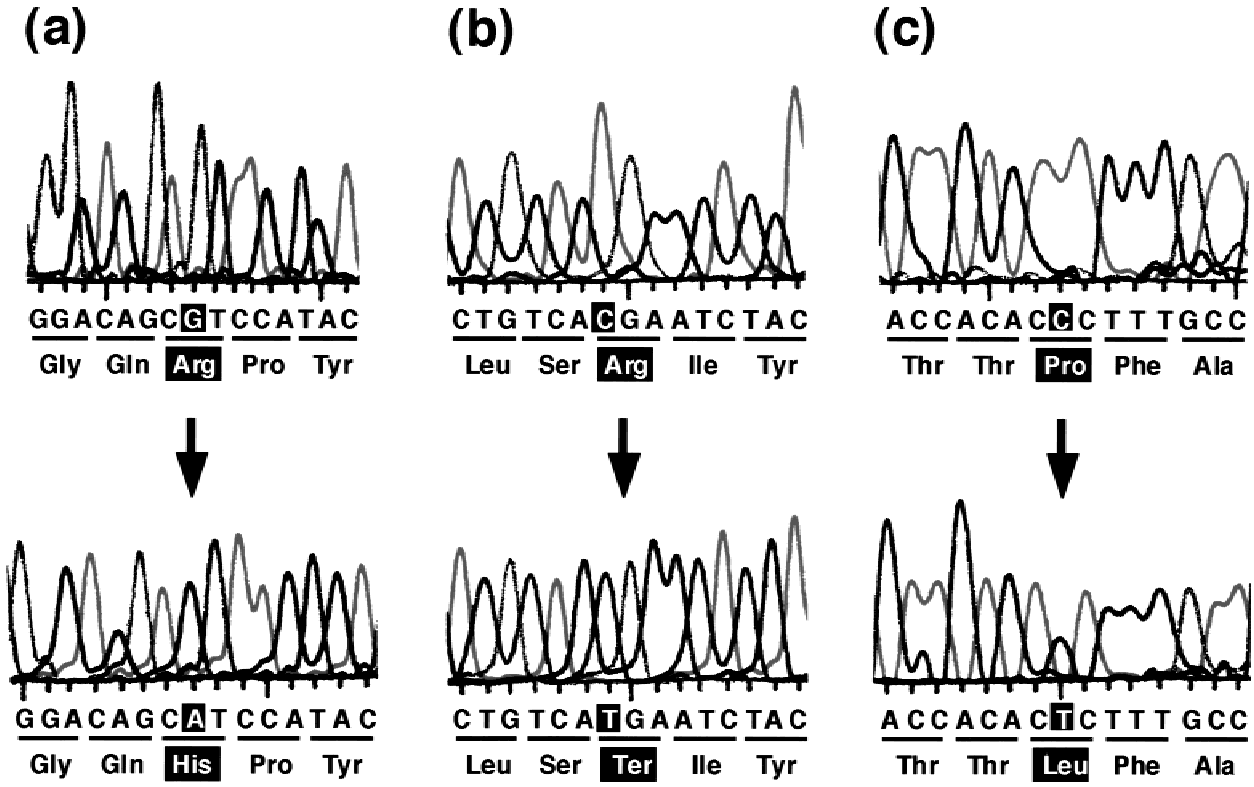

Fig. 1. Nucleotide sequences of missense and nonsense mutations identified in patients with GSD-Ia. **a:** Single base substitution of "g-to-a" at nt 327, resulting in an amino acid change of arginine (83) -to-histidine. **b:** "c-to-t" substitution at nt 588, causing an amino acid change of arginine (170)-to-termination codon. **c:** "c-to-t" substitution at nt 849, causing a proline (257)-to-leucine substitution.

is associated with a more severe phenotype of GSD-Ia. Since R170X is predicted to truncate G6Pase significantly, it unlikely retains enzymatic activity. The in vitro expression study confirmed that *G6PC*-cDNA carrying R170X produced no residual activity. As R170X was identified in both parents of patient 1 and in patient 4 who is unrelated to patient 1, it is most likely that the mutation is prevalent among Japanese GSD-Ia patients. Interestingly, R170X was also reported recently in a German patient (a homozygote) and two Dutch patients (compound heterozygotes) [Rake et al., 1999]. This suggests recurrent R170X mutations arose among different ethnic populations at a hypermutable CpG dinucleotide at nt 588 in *G6PC*.

Patient 3 is a homozygote for P257L. She presented with the mildest symptoms among the four patients, and a diagnosis of GSD-Ia was not made until adulthood. She had never been placed on dietary therapy and had no hypoglycemic episodes. According to a nine-transmembrane helical model of G6Pase, the Pro-257 residue is located in loop 3L between helices 6 and 7 [Hemrika and Wever, 1997; Pan et al., 1998]. Loop 3L, which consists of 33 amino acid residues, is located on the luminal side of the endoplasmic reticulum, although its functional role has remained unknown. P257L is a second mutation identified in this region. The other mutation reported in this loop is W236R, which retained 4% of normal G6Pase activity [Lei et al., 1995a]. Although the clinical profile of the patient who had W236R has not been provided, the residual G6Pase activity of W236R (4%) and P257L (1.2%) and the association of P257L with a mild clinical phenotype suggest that the structural requirement of loop 3L for the catalytic activity of the enzyme is less critical than that of the transmembrane helices.

The R83H, seen in patient 2, was previously reported to be a prevalent mutation among Chinese patients [Hwu et al, 1995]. The geographical proximity of the two countries raises the possibility that the mutation might have the same ancestral origin. However, the R83H allele in patient 2 is associated with the 1176T polymorphic allele, whereas R83H in Chinese patients was in linkage disequilibrium with the 1176C allele [Wong et al., 1998]. The different haplotype between the two patients may favor that two mutations occurred independently at a CpG dinucleotide. According to the nine-transmembrane helical model of G6Pase, Arg-83 resides on the luminal surface of the endoplasmic reticulum membrane [Hemrika and Wever, 1997]. Structure-function studies have suggested that the Arg-83 residue is involved in stabilizing the phosphoryl-enzyme intermediate formed during catalysis and contributes to the active center of the enzyme [Lei et al., 1995b]. The importance of the Arg-83 residue is further supported by the identification of another disease-causing substitution at the same position, R83C, in Caucasian and Turkish patients [Lei et al., 1993; Hüner et al., 1998].

The g727t mutation creates an aberrant 3′-splicing site within *G6PC* exon 5 [Kajihara et al., 1995]. Consequently, a transcribed mRNA from the mutant allele

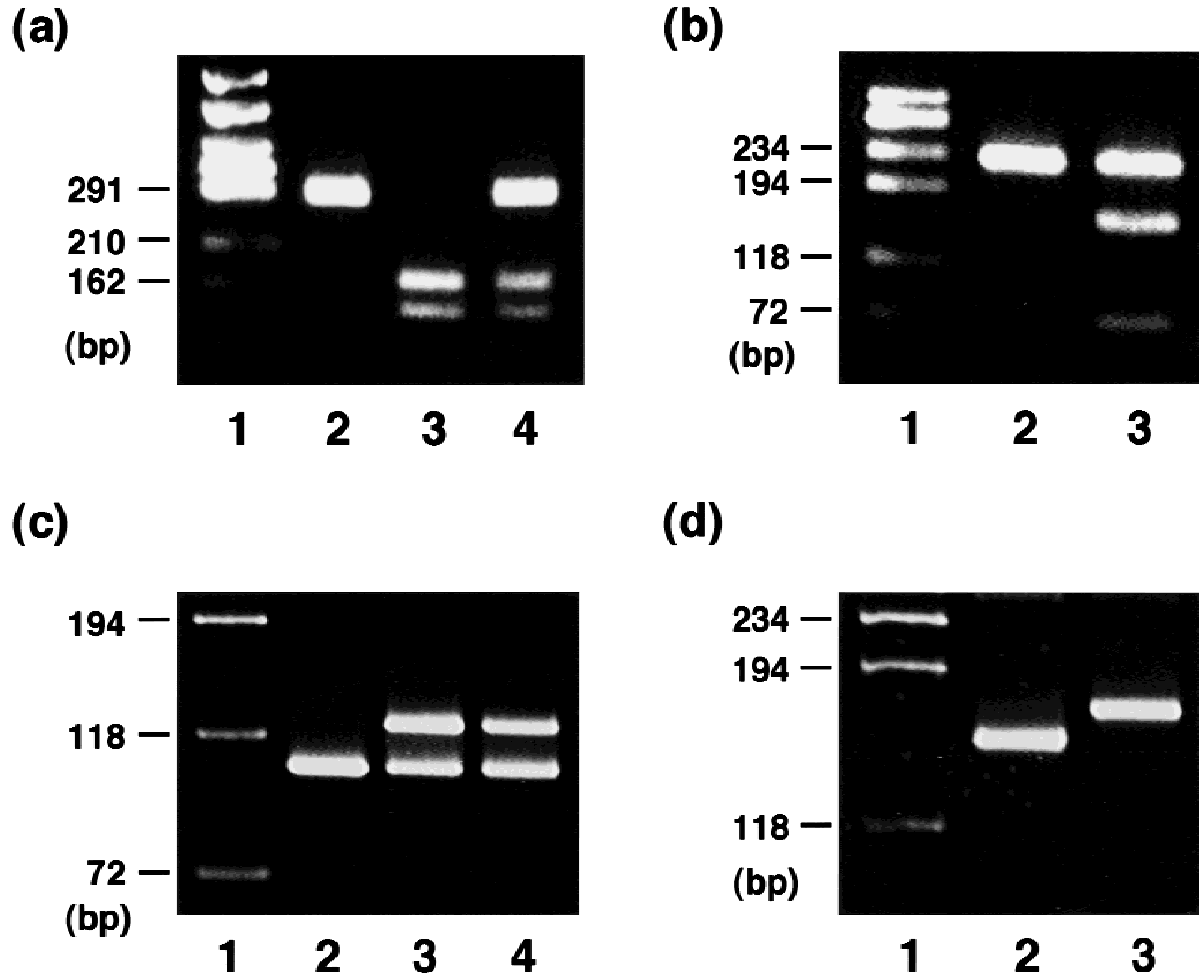

Fig. 2. Detection of R170X, R83H, g727t, and P257L by restriction analysis with *Bsp*HI (**a**), *Fok*I (**b**), *Rsa*I (**c**), and *Eco*NI (**d**), respectively. **a:** Lane 1, size marker; lane 2, a homozygote for the wild-type allele (293 bp); lane 3, a homozygote (patient 3) for R170X (163 bp + 130 bp); lane 4, a heterozygote (patient 4). **b:** Lane 1, size marker; lane 2, a normal subject; lane 3, a heterozygote for R83H (patient 2). **c:** Lane 1, size marker; lane 2, a normal subject; lane 3, a heterozygote for g727t (patient 2); lane 4, a heterozygote (patient 4). **d:** Lane 1, size marker; lane 2, a normal subject; lane 3, a homozygote for P257L (patient 3).

carries 91-nucleotide deletion in exon 5, producing a severely truncated protein of 201 amino acids. This mutation was previously reported as the only predominant mutation in Japanese patients [Kajihara et al, 1995; Okubo et al., 1997]. However, the result of our study implicates that there are indeed other mutations in Japanese patients and that the incidence of g727t is not as high as previously reported.

TABLE I. Expression Studies of R83H, R170X, and P257L Mutations in COS7 Cells

Mutation in expression vector	G6Pase activity[a] Mean ± SD (n = 3) nmol Pi/mg protein/min	% Wild type
Wild type	157.9 ± 55.8	100
R83H	0.86 ± 1.38	0.5
R170X	0.45 ± 0.33	0.3
P257L	1.87 ± 0.70	1.2

[a]The activity in mock transfection (5.94 ± 0.17) was subtracted.

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